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Milk Lipase (Tributyrinase) in the Milk of Cows on Protein-free Feed

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34 **Lipase in Milch** (bei proteinfreier Fütterung)

Milchwissenschaft

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Lipase and lipolysis in milk have been the subjects of recent reviews (CHANDAN & SHAHANI 1964, SCHWARTZ 1965), and LAWRENCE 1967) has summarised work on microbial lipases in dairy products. There are many reports about the characteristics of the lipase or lipase system in whole milk or skimmilk. The very varied resulds are not surprising considering the complexity of these systems. However, a few general conclusions relevant to the method described below can be drawn.

1. Temperature changes in and agitation of the milk may affect the activity of the enzyme system.
2. Deactivation of the enzyme system when the milk is stored may be reduced or eliminated by treating it with nitrogen or hydrogen sulphide, bubbled through the milk. This treatment also counteracts the effects of light, which depresses lipase activity in milk.
3. Pasteurisation at low or high temperatures normally inactivates the enzyme system almost completely.

There has been little attempt to standardise the measurement of milk lipase, and the number of variations of the reaction conditions governed by substrate, temperature etc., together with methods for determining the lipolysis, or hydrolysis, produced is almost equal to the number of authors. Some authors have acknowledged the recommendations of the International Union of Biochemistry (Commission on Enzymes 1961), according to which

(a) enzyme assays should be based wherever possible upon measurements of initial rates of reaction, with conditions of the standard assay arranged so that its kinetics approach zero order, and

(b) one enzyme unit (U) is defined as that amount which will catalyse the transformation of one micromole of the substrate per minute. The unit of specific activity will then be U/mg protein and the unit of concentration U/ml.

Thus when lipase activity is measured by titration with alkali the activity unit may be defined as that amount catalysing the release of one microequivalent of acid per minute.

The method selected in this laboratory for the measurements of milk lipase was the pH-stat procedure of MARCHIS-MOUREN et al. (1959), which is direct and rapid. The pH of the reaction mixture is kept at its optimum value by continuous addition of measured volumes of standard alkali, and the enzymatic activity is found from the slope of the titration-time graph. PARRY et al. (1966) present a convenient form of this method, though two of these authors earlier stated (CHANDAN & SHAHANI 1963a) that it is inapplicable to whole milk or crude enzyme products prepared from it.

The substrate used in the assays reported herewith was tributyrin. Previously reported data obtained when this substrate was used are summarised in table I. An average value, in many cases approximate, was derived in each case and calculated in terms of the activity unit U.

Table 1. Reported milk lipase concentrations in determinations using tributyrin as substrate. U, the unit of activity, is one microequivalent of acid liberated per minute

Authors	Reaction conditions			Lipase concentration U/ml
	pH	Temp. °C	Time	
Downey & Andrews (1966)	8.5	25	5 min.	0.9
Dunkley & Smith (1951a)	8.7	37	30 min.	0.5
Dunkley & Smith (1951b)	8.7	37	30 min.	0.6
Frankel & Tarassuk (1956)	8.8	37	15 min.	1.4
Kelly (1945)	unbuffered	37	24 hr.	0.004
Mattick & Kay (1938)	8.4—8.6	37	6 hr.	0.1
Nelson & Jezeski (1955)	8.5	38	1 hr.	0.09
Peterson et al. (1943)	8.5	40	30 min.	0.8
Smith et al. (1949)	8.8—8.4	37	30 min.	0.6
Stadhouders & Mulder (1964)	8.8	15	1.5 hr.	1.1
Tarassuk & Frankel (1957)	8.8	37	15 min.	1.0

The mean of the concentration figures in table 1 is 0.6 U/ml.

In recent years fundamental knowledge about the nature of milk lipase has been gleaned (CHANDAN & SHAHANI 1963 a, b, DOWNEY & ANDREWS 1965 a, b, 1966, FOX et al. 1967 and SHAHANI & CHANDAN 1965). These

workers have confirmed that there are several distinct lipases in milk, the major components probably being adsorbed on the casein micelles, or else being part of the $\varkappa$-casein complex. Using milk clarifier slime as the source, CHANDAN and SHAHANI (1963a, b) isolated an enzyme product whose purity and homogeneity were established by ultracentrifugal and gel electrophoretic techniques. With milk fat as the substrate the optima of the enzyme were pH 9.0—9.2 and about 37 ° C. Each milk constituent was found to have its own characteristic effect on the enzyme activity: some proteins inhibited the enzyme whereas others at low concentration stimulated it so that it is to be expected that its activity in whole or skim-milk is the net results of the opposing effects (SHAHANI & CHAHAN 1965). Moreover, DOWNEY and ANDREWS (1965 b) have shown that the non-enzyme proteins of milk can show considerable esterase activity. In studies using p-nitrophenyl acetate as the substrate the activity of such proteins accounted for some 70 % of the total esterase activity of milk. These authors point to the possible fault in interpreting the activity in whole milk, skim milk or unpurified enzyme sources as being due entirely to enzymes.

Experimental

Milk samples. (i) Samples from individual Ayrshire cows, farm 1. The cows had each been on a purified, protein-free feed for a considerable period. The experiment was started in 1961 and is still in progress, and altogether seven cows have been adapted to the test feed. A good yield of milk of composition close to that of milk of normally-fed cows has been maintained over a period covering, for some of the test animals, several successive lactations (VIRTANEN 1966). The test milk proteins are quite normal (SYVÄOJA & VIRTANEN 1965), and the peroxidase, alkaline phosphatase, xanthine oxidase, aldolase, catalase and α-amylase concentrations resemble those of normal milk (SYVÄOJA & VIRTANEN 1967).

(ii) Herd milk, Ayrshire cows on normal stall feed or pasture, farm 1. Referred to as control milk 1.

Samples (i) and (ii) were cooled to about 20 ° C after milking and were transported to the laboratory in full vacuum flasks. The lipase assay was performed immediately, the interval between milking and assay thus being 4—6 hours.

(iii) Ayrshire cows, herd milk and milk from individual cows, normal winter rations, farms 2 and 3. These samples were transported to the laboratory in polythene bottles, with no control of temperature or agitation en route. Lipase assay was performed immediately upon receipt, or after storage of the whole milk or skim milk under nitrogen overnight at about +3 ° C, nitrogen gas having been bubbled through the samples to remove oxygen. These samples are referred to collectively as control milk 2.

Lipase measurement. The pH-stat apparatus consisted of a 37—38 ° C water bath, magnetic stirrer, pH meter (Beckman Zeromatic II), nitrogen supply and 5 ml burette (0.01 ml graduations, teflon tap) containing approximately 0.04 n KOH solution standardised against 0.01 n HCl at the time of the test. pH meter drift over a period of 10 minutes was checked at room temperature in phosphate buffer, pH 6.9 before and after the assays, and was found to be negligible every time.

The milk sample was centrifuged (10 mins. at 3000 xg) at 15—20 ° C and the skim milk, containing re-incorporated sediment obtained during the centrifugation, collected and pipetted (15 ml) into an 18 x 180 mm test tube, which was brought to 37 ° C by immersion in the water bath for 10 minutes. 5 ml tributyrin was pipetted into the tube, which was stoppered and shaken vigorously by hand 5 seconds. The emulsion was poured into a 100 ml beaker containing 15 ml water at 37 ° C and the test tube was washed out with 5 ml water, this also being added to the beaker. The contents of the beaker were stirred continously with a magnet, nitrogen was layered over the liquid surface at the rate of about 100 ml/min., and the pH of the reaction mixture was monitored. The pH was then adjusted rapidly and accurately to 8.8 with dropwise addition of 0.25 and 0.1 n KOH

and the time zeroed. The pH was maintained at 8.8 by continuous, dropwise addition of the 0.04 n KOH and burette readings were taken every minute for 10 minutes.

A blank test was made on a further 15 ml portion of the test sample by immersing it in an 80 ° C water bath for 3 minutes, cooling to 37 ° C, emulsifying and performing the titration as before. The blank readings were subtracted from the test readings and the remainders, corrected to 0.040 n alkali, plotted on a graph versus time. The rate of hydrolysis during the reaction period was constant, and thus the slope of the titre-time curve was a measure of the lipase concentration, which was quoted in terms of micro-equivalents of acid liberated/ml skim milk/minute.

The lipase assay method was evaluated in a series of tests:

(i) Figure 1 shows the blank titration curve B (mean of 22 runs) in relations to the overall curve T (72 runs) and the net enzymatic activity curve N.

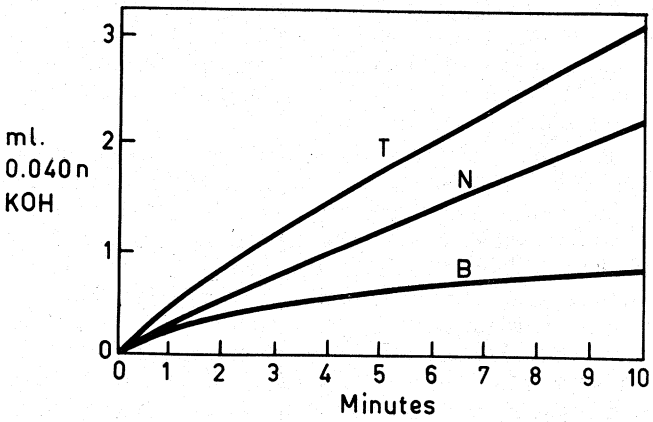

Figure 1. pH-stat milk lipase assay: mean overall T, blank B and net N titre-time curves.

The blank value accounts for about 50 % of the overall value after 1 minute, this proportion falling to 27 % after 10 minutes. The blank values were unaffected by more rigorous heating (100°C, 5 min.) and

they were reproducible at intervals of weeks or months so that in practice blanks were not run with every batch of test samples. The same blank values were obtained with reconstituted skim milk, prepared by dispersing 10 g powder (Kuivamaito Oy, fat less than 0.5 % in dry matter) in 100 ml water, either fresh or previously heated as for the preparation of the blanks, and only slightly higher values were found when the volume of the reconstituted skim milk was increased from 15 to 30 ml.

The blank titration curve seemed thus to be a valid estimate of the nonenzymatic formation of acid contributing to the overall titration curve of the fresh skim milk sample.

(ii) The results of duplicate runs are tabulated below. Between runs the samples were held in stoppered tubes in a cupboard at room temperature. The activity unit U is one microequivalent of acid liberated per minute.

	Lipase concentration, U/ml skim milk					
Sample	1	2	3	4	5	6
First run	0.43	0.96	0.53	0.29	0.72	0.27
Second run	0.44	0.95	0.53	0.31	0.71	0.27

(iii) The effect of shaking used to disperse the substrate on the enzyme activity was checked. In addition to the test skim milk, 15 ml portions of the reconstituted skim milk, prepared as described above, were used in the reaction mixture. A comparison was made between the lipase concentration with the tributyrin emulsified in the test sample and the concentration with the tributyrin emulsified in the reconstituted skim milk powder. The results were as follows:

	Lipase concentration, U/ml			
Sample	1	2	3	4
Tributyrin dispersed in test sample	0.96	0.50	0.75	0.64
Tributyrin dispersed in reconstituted skimmilk	1.01	0.54	0.77	0.64

Thus it appeared that this simple substrate dispersion method, while being adequate for saturation of the enzyme, had an inappreciable effect on the rate of lipolysis as measured under these conditions.

The effect of more vigorous agitation on lipolysis, using the sample's own milk fat as substrate, was examined. 13 test milk samples were taken during the period February-June 1967. The methods were, essentially, those of JENSEN et al. (1960): acid degree values (ADV) of the milk fat of the sample were measured (by the silica gel method of HARPER et al. 1956) initially, after incubation under N_2 at about +3 ° C for 24 hr. (spontaneous lipolysis) and after the same incubation following 30 seconds' stirring (Hamilton Beach No. 30, lower speed) (induced lipolysis). The mean initial ADV found was 0.6 (standard error ± 0.1); after spontaneous lipolysis the ADV was 1.9 (± 0.4) and after induced lipolysis 5.6 (± 1.3). These figures, which are very similar to those reported by JENSEN et al. for a herd of 10 Holstein cows on normal winter rations or pasture, illustrate the frequently found effect of agitation on lipolysis in milk.

RESULTS

Table 2. Lipase (tributyrinase) concentration of the milk of cows on a protein-free feed (test milk) and of herd milk of normally-fed cows (controls 1 & 2). U, the unit of activity, is one microequivalent of acid liberated per minute

	Test Milk				Controls	
Cow	Eiru	Aino	Jairu	Metta	1	2
Number of samples	3	20	17	19	20	16
Lipase concentration, U/ml	0.61	0.60	0.70	0.50	0.63	0.63
Lipase concentration, standard error ±	—	0.05	0.03	0.04	0.05	0.04
Mean concentration			0.60		0.63	

Thus there was considerable variation in the milk lipase activity of milk from both individual test cows on the protein-free feed and in the activity of the control milks. There were no clear trends in activity levels with season or stage of lactation, and the activity of the test milk is normal, considering the values of the control milks found here and those reported elsewhere (Table 1). This finding is consistent with the normal activities of other test milk enzymes studied in this laboratory (SYVÄOJA & VIRTANEN 1967).

The average control milk activity was the same

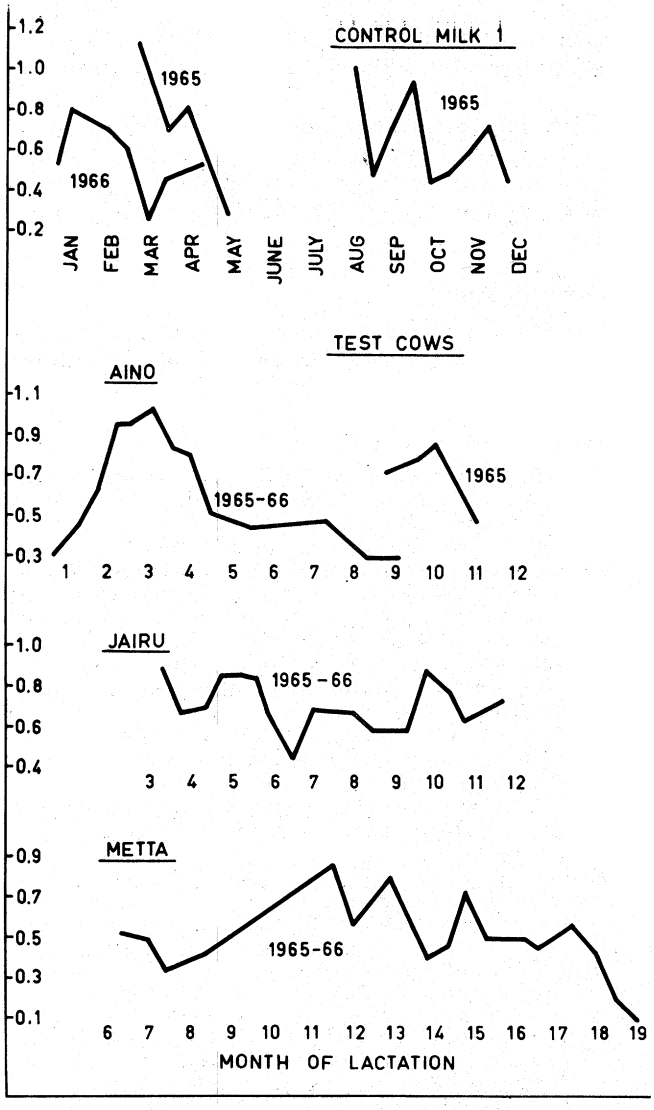

Figure 2. Milk lipase concentration: variation in the milk of individual cows (test cows) on protein-free feed, and in herd milk of normally-fed cows (control milk 1). Units: microequivalents of acid liberated/ml skimmilk/min.

whether the samples were protected frgm temperature changes, light and agitation during transportation or not.

Mean approximate specific activity figures calculated from the lipase concentration and the whole milk protein concentration were in the range 11—20 microequivalents of acid liberated per minute per g protein.

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34 Lipase in Milch (bei proteinfreier Fütterung).

Während eines Jahres wurden Milchproben von Ayrshire-Kühen gesammelt und der Lipasegehalt nach der pH-Methode gemessen. Die Untersuchung wurde wie folgt durchgeführt: 5 ml Tributyrin wurden in 15 ml der zu untersuchenden Magermilch emulgiert, dann wurden 20 ml Wasser zugegeben. Durch Tritration mit Kalilauge wurde der pH auf 8,8 eingestellt, bei 37—38 ° C wurde 10 Min. bebrütet. Von dem erhaltenen Lipasewert wurde der Blindwert abgezogen, der durch Titration 3 Min. auf 80 ° C erhitzter Magermilch erhalten wurde.

Die Lipaseaktivität wurde ausgedrückt in Einheiten der Mikroäquivalente der je Minute freigesetzten Säure. Die Hauptlipasekonzentration in Milch von 3 Kühen, die proteinfrei gefüttert wurden, war 0,6, 0,7 und 0,5. Der Lipasegehalt der Milch von normal gefütterten Herden betrug 0.63. Die Proteinfütterung hatte keinen Einfluß auf den Lipasegehalt. Dok.-Ref.

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34 Lipase in milk (protein-free feeding).

Milk samples were collected from Ayrshire cows over a period of one year, and lipase was measured by a pH-stat method. Reaction details: 5 ml tributyrin emulsified in 15 ml skimmilk under test, 20 ml water, 37—38 ° C, pH 8.8 maintained by titration with 0.04n KOH, 10 min. reaction period. Blank values determined with heated skimmilk (80 ° C 3 min.) were substracted from the overall values to give the net enzymatic activity curve. The kinetics of the enzymatic hydrolysis were zero order, the slope of the activity curve being the lipase activity, which was expressed in units (U) of micro-equivalents of acid liberated per minute. Mean lipase concentrations of milk from three cows receiving the protein-free feed were 0.60 (20 samples), 0.70 (17) and 0.50 (19) U/ml skimmilk, and of herd milk from normally fed cows 0.63 U/ml (20 samples).

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